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- 7 MAR 2002
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1/77

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Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet, from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
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1. Your reference — JPP141

2. Patent application number *(The Patent Office will fill in this part)* — **0205378.3** — 07 MAR 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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Patents ADP number *(if you know it)* 7547193001

If the applicant is a corporate body, give the country/state of its incorporation — ENGLAND

4. Title of the invention — Bacterial Spores

5. Name of your agent *(if you have one)* — Barker Brettell

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)* 7442494002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of Filing *(day/month/year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day/month/year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d)) — YES

BACTERIAL SPORES

This invention relates to the use of spores in eliciting an immune response, a method of eliciting said immune response and to a method of making said spores.

Infection is the leading cause of death in human populations. The two most important contributions to public health in the past 100 years have been sanitation and vaccination, which together have dramatically reduced deaths from infectious disease.

The development of improved vaccination strategies has always been of the utmost importance for a number of reasons.

Firstly, to provide better levels of immunity against pathogens which enter the body primarily through the mucosal surfaces. Vaccines are generally given parentally. However, many diseases use the gastrointestinal (GI) tract as the primary portal of entry. Thus, cholera and typhoid are caused by ingestion of the pathogens *Salmonella typhi* and *Vibrio cholera* and subsequent colonisation at (*V. cholera*) or translocation (*S. typhi*) across the mucosal epithelium (lining the GI tract). Similarly, TB is initially caused by infection of the lungs by *Mycobacterium tuberculi*. Immunisation via an injection generates a serum response (humoral immunity) which includes a predominant IgG response which is least effective in preventing infection. This is one reason why many vaccines are partially effective or give short protection times.

Secondly, to provide needle-less routes of administration. A major problem of current vaccination programmes is that they require at least one injection (for example tetanus vaccine). Although protection lasts for

10 years, children are initially given three doses by injection and this should be followed by a booster every 5 years. In developed countries many people will choose not to take boosters because of 'fear of injection'. In contrast, in developing countries where mortality from tetanus is high the problems lie with using needles that are re-used or are not sterile.

Thirdly, to offer improved safety and the minimisation of adverse side effects. Many vaccines consist of live organisms which are either rendered non-pathogenic (attenuated) or are inactivated in some way. While in principle, this is considered safe there is evidence showing that safer methods must be developed. For example, in 1949 (the Kyoto incident) 68 children died from receiving a contaminated diphtheria vaccine (Health 1996). Likewise, in the Cutter incident of 1995, 105 children developed polio. It was found that the polio vaccine had not been correctly inactivated with formalin. Many other vaccines, for example the MMR (measles-mumps-rubella) vaccine and the whooping cough vaccine (Health, 1996) are plagued with rumours of side effects.

Fourthly, to provide economic vaccines for developing countries where poor storage and transportation facilities prevent effective immunisation programmes. In developing countries where a vaccine must be imported it is assumed that the vaccine will be stored and distributed correctly. The associated costs of maintaining vaccines in proper hygienic conditions under refrigeration are significant for a developing country. For some vaccines such as the oral polio vaccine and BCG vaccine the vaccines will only survive for one year at 2-8°C (Health, 1996). The need for a robust vaccine that can be stored indefinitely at ambient temperature is a high priority now for developing countries. This type of vaccine should ideally be heat stable, able to withstand great variations in temperature as well as desiccation. Finally, a vaccine that is simple to

produce would offer enormous advantages to a developing country and would potentially be producable in that country.

A way of ameliorating these problems has been sought.

Accordingly, the present invention provides a spore genetically modified with genetic code comprising at least one genetic construct encoding an antigen and a spore coat protein as a chimeric gene, said genetically modified spore having said antigen expressed as a fusion protein with said spore coat protein.

It is an advantage of the present invention in that the use of spores to administer vaccines will eliminate the need for injections and the problems associated with needles in developing countries. In addition to this, spores are stable and are resistant to heat and desiccation, therefore overcoming problems of storing vaccines in developing countries. Spores are easy to produce, and can be done at low cost making the production of vaccines in accordance with the invention economical and finally, as a non-pathogen and its current use as an oral probiotic, the use of *Bacillus subtilis* makes this a safer vaccine system than those currently available.

It is a further advantage of the invention that the spores elicit an immune response at the mucosal membranes. This makes the vaccination more effective against mucosal pathogens e.g. *S.typhi, V.cholera* and *M.tuberculi*.

A vaccine delivered at the mucosal surfaces will be more effective in combating those diseases which infect via the mucosal route. The mucosal routes of vaccine administration would include oral, intra-nasal and/or rectal routes.

Preferably the spore is of Bacillus species.

Preferably the vegetative cell is of Bacillus species.

The genetic code comprises DNA or cDNA. It will be appreciated that the term 'genetic-code' is intended to embrace the degeneracy of codon usage.

The genetic construct preferably comprises at least part of a spore coat protein gene and at least part of an antigen gene, in the form of a chimeric gene.

The antigen gene is preferably located at the 3' end of the spore coat protein gene. Alternatively the antigen gene may be located at the 5' end of the spore coat protein gene or internally of the spore coat protein gene.

Preferably the genetic construct comprises a spore coat promoter at the 5' end of the chimeric gene.

The genetic construct comprises a plasmid or other vector wherein the chimeric gene is located in a multiple cloning site flanked by at least part of an *amyE* gene. Alternatively, the genetic construct comprises a plasmid or other vector wherein the chimeric gene is located in a multiple cloning site flanked by at least part of a *thrC* gene. It will be appreciated that the invention is not limited to insertion at *amyE and thrC* genes. Insertion into any gene is permissible as long as the growth and sporulation of the organism is not impaired i.e. the insertion is functionally redundant.

Preferably the genetic construct is used to transform a vegetative mother cell by double crossover recombination. Alternatively the genetic

construct is an integrative vector e.g. p JH101 which is used to transform the vegetative mother cell by single crossover recombination.

The antigen is preferably at least one of tetanus toxin fragment C or labile toxin B subunit. Alternatively the antigen may be any antigen, adapted, in use, to elicit an immune response.

The spore coat protein is preferably *cotB*. Alternatively the spore coat protein is selected from the group consisting of *cotA, cotC, cotD, cotE* and *cotF*. Alternatively the spore coat protein is selected from the group consisting of *cotG, cotH, cotJA, cotJC, cotM, cotSA, cotS, cotT, cotV, cotW, cotX, cotY* and *cotZ*.

The spores may be administered by an oral or intranasal or rectal route. The spores may be administered using one or more of the said oral or intranasal or rectal routes.

Oral administration of spores may be suitably via a tablet a capsule or a liquid suspension or emulsion. Alternatively the spores may be administered in the form of a fine powder or aerosol via a Dischaler® or Turbohaler®.

Intranasal administration may suitably be in the form of a fine powder or aerosol nasal spray or modified Dischaler® or Turbohaler®.

Rectal administration may suitably be via a suppository.

The spores according to the invention are inactivated prior to administration such that they do not germinate into vegetative cells. Preferably they are heat inactivated.

According to a further aspect the present invention provides a genetically modified spore according to the invention for use as an active pharmaceutical substance.

According to a further aspect the present invention provides at least two different genetically modified spores, the or each modified spore expressing at least one different antigen, according to the invention for use as active pharmaceutical compositions.

According to a further aspect, the present invention provides a method of producing a genetically modified spore, which method comprises the steps;

producing genetic code comprising at least one genetic construct encoding an antigen and a spore coat protein as a chimeric gene;

using said at least one genetic construct to transform a vegetative mother cell;

inducing said transformed mother cell to sporulate;

isolating the resulting genetically modified spores.

The spores are inactivated prior to administration such that they do not germinate into vegetative cells. Preferably they are heat inactivated.

According to a further aspect, the present invention provides a composition comprising a genetically modified spore, according to the invention, in association with a pharmaceutically acceptable excipient or carrier.

Suitable pharmaceutically acceptable carriers would be well known to a person of skill in the art and would depend on whether the pharmaceutical composition was intended for oral, rectal or nasal administration.

According to a further aspect the present invention provides a genetically modified spore according to the invention for use in a method of medical treatment.

According to a further aspect, the present invention provides a genetically modified spore according to the invention for use in the manufacture of a medicament, for use in a method of medical treatment.

A method of medical treatment is preferably immunising a human or animal against a disease by administering a vaccine.

According to a further aspect, the present invention provides a method of medical treatment, which method comprises the steps of;

orally or intra-nasally or rectally administering a genetically modified spore according to invention to a human or animal in need of medical treatment;

said genetically modified spore eliciting an immune response for use in the prevention of a disease.

The invention will now be described, merely by way of example.

Chimeric genes were constructed in which TTFC or LTB gene sequences were fused, in frame, to a specific *cot* gene. The constructs were then introduced into the chromosome of *B.subtilis*. Expression of the chimeric genes was then confirmed and immunisations were performed using

inbred mice (Black C57 inbreds). Immune responses were then measured. Unless otherwise stated, *cot* genes refers to *cotA, cotB, cotC, cotD, cotE* and *cotF*.

Table 1: Recombinant chimeric genes

TTFC[1]	LTB[2]	TTFC & LTB
cotA-TTFC	*CotA*-LTB	*cotA*-LTB *cotB*-TTFC
cotB-TTFC	*CotB*-LTB	*CotA*-LTB *cotE*-TTFC
cotC-TTFC	*CotC*-LTB	*cotA*-LTB *cotD*-TTFC
cotD-TTFC	*CotD*-LTB	
cotE-TTFC	*CotE*-LTB	
cotF-TTFC	*CotF*-LTB	

[1]placed at the *amyE* locus

[2]placed at the *thrC* locus

a) Construction of Chimeric Genes

PCR (polymerase chain reaction) was used to amplify the specific *cot* gene to enable the 3'-end of the amplified *cot* gene sequence to be fused to the 5'-end of a similar PCR product carrying the 5'-end of TTFC or LTB. Ligation PCR products was achieved by restriction digest of the PCR products. This was enabled by PCR amplification using primers carrying embedded restriction sites. Appropriate cloning vectors (see below) were restricted (cut) with restriction enzymes recognising the 5'-end of the *cot* gene and the 3'-end of the antigen gene. The cleaved PCR products were ligated with cleaved vector and recombinants assessed using standard techniques known to those in the art.

(In this process it is essential that the *cot* gene carries its own promoter sequences at the 5'-end of the gene.)

b) Vectors for chromosomal insertion

The essential features of the vector pDG364 are the right and left flanking arms of the *amyE* gene (referred to as *amyE* front and *amyE* back). Cloned DNA (i.e. the *cot*-antigen chimera) is introduced into the multiple cloning sites using general PCR techniques, the clone is then validated and the selected plasmid clone linearised by digestion with enzymes recognising the relevant backbone sequences (e.g. *Pst*I). The linearised DNA is now used to transform competent cells of *B. subtilis*. Transformants are selected by using an antibiotic resistance gene carried by the plasmid (chloramphenicol resistance). The linearised plasmid will only integrate via a double crossover recombination event using the front and back flanking arms of *amyE* for recombination. In the process the cloned DNA is introduced into the *amyE* gene and the *amyE* gene inactivated in the process. This procedure minimises damage to the chromosome and does not impair cell growth, metabolism or spore formation. Since the inserted gene chimera is at the *amyE* locus in the chromosome the gene is in *trans* to the normal *cot* genetic locus. For example, when the *cotA* gene is fused to TTFC and introduced into the *amyE* locus, there also exists a normal *cotA* gene elsewhere in the chromosome. Thus, the cell is now partially diploid, it carries one normal *cotA* gene and one chimeric gene.

In addition to pDG364, another suitable vector is pDG1664. This vector is almost identical to pDG364 but differs by the following;

i) it carries the erythromycin-resistance gene, *erm*. This enables selection of transformed *B.subtilis* cells using erythromycin instead of chloramphenicol, and

ii) instead of the front and back portions of the *amyE* gene it carries the front and back portions of the *thrC* gene. *thrC* is redundant.

A final route for cloning is to use an integrational vector. Many such vectors exist, but the best is pSGMU2 or pJH101. In this method, the *cot* gene in the clone and the resident chromosomal cot gene would introduce a cot-antigen chimera into the chromosome by virtue of homology shared. Following single crossover recombination the entire plasmid with the *cot*-antigen chimera is introduced into the chromosome at the chromosomal position of the *cot* gene. Thus, in doing so, the resident *cot* gene is modified. This is in contrast to the pDG364/pDG1664 vectors which are placed elsewhere and do not modify the resident *cot* gene.

c) Multiple antigen presentation

To achieve multiple antigen presentation on the spore coat it is necessary to use two different plasmid vectors, for example pDG364 and pDG1664 . One chimeric gene is made in pDG364 and the chimera introduced at the *amyE* locus and a second chimera made in pDG1664 and introduced at the *thrC* locus. In this case each transformational event requires separate antibiotic resistance selection. It will be appreciated that any relevant technology known to those of skill in the art could be applied to create multiple antigen presentation on the spore coat.

d) Validation of strains

Isogenic strains carrying the chimeras shown in Table 1 were validated for expression of a foreign antigen. Specifically, strains were grown and induced to sporulate using established procedures. Spores at about hour 20-24 following the induction of sporulation were harvested and total spore coat proteins recovered using ether SDS-DTT extraction or NaOH extraction. Western blotting using anti-TTFC or anti-LTB antibodies was

used to demonstrate the presence of the foreign antigen. Levels of protein were generally lower in the *cotE* and *cotF* chimeras. The validation confirmed that these antigens were not subject to inadvertent proteolysis or degradation.

TTFC can be expressed at the *thrC* locus and LTB from the *amyE* locus with identical levels of gene expression.

Final validation of strains involved assessing whether the spore's resistance properties had been affected in any way. Spore suspensions of each strain were prepared (shown in Table 1). These spore suspensions were heated at 80°C for 30 min and shown to carry approximately the same number of viable spore units before and after heat treatment. The expression of the foreign antigen had no effect on spore resistance properties.

e) Intra-Peritoneal Immunisation

Spores were prepared from each of the recombinant strains shown in table 1 and the suspensions were purified by repeated washing to remove contaminating vegetative cells. The suspensions were then heat-treated at 65°C to inactivate any residual vegetative (unsporulated cells) and subsequently used to dose mice via an intra-peritoneal route at a dose of 1×10^9 spores/ml on days 0, 14 and 28. Serum samples were taken thereafter and antibody titres determined by ELISA. All constructs gave high levels of serum IgG compared to naïve mice or mice immunised with non-recombinant spores. These results showed that both TTFC and LTB chimeras are immunogenic and are capable of eliciting an immune response.

f) Mucosal immunity

To achieve mucosal immunity two approaches were used; oral dosing and intranasal dosing. For oral administration of spores expressing TTFC fusion proteins, 1 X 10^{10} spores/dose were administered by intra-gastric lavage to black C57 inbred mice using multiple doses over a 35 day period. Tail bleeds and fecal samples were taken at appropriate times and analysis made for serum IgG in tail bleeds and IgA in fecal samples. High levels of anti-TTFC IgG and IgA were found. Similar high levels of immunity (both IgG and IgA) following oral immunisation of mice with spores expressing LTB (not shown) have been observed.

Similarly, intranasal dosing of mice with spores expressing LTB was achieved using 1 X 10^9 spores/dose using micropipettes to administer spores (20μl) on days 0, 14 and 28. High levels of mucosal immunity were generated demonstrating the potential of spores as mucosal vaccine vehicles using the intra-nasal route for delivery. We have observed similar high levels of immunity (both IgG and IgA) following intranasal immunisation of mice with spores expressing TTFC.

Using spores expressing both TTFC and LTB we were able to achieve similarly high levels of anti-TTFC and anti-LTB IgG and IgA following oral and intranasal immunisation.

g) dosage

In pilot studies we know that about 1 X 10^9 spores/dose is the minimum dose of spores required for oral immunisation and 1 X 10^8 spores/dose for intranasals. It is possible that with alternative dosing regimes (of which there are many) a lower dose could be used.

Spores according to the invention could be used to display any biologically active molecule. For example, an enzyme for an industrial application.

Any spore forming species could be used for heterologous antigen presentation. However, other spore-forming micro-organisms are unlikely to carry the same complement of spore coat proteins. Indeed, some spore formers such as *Bacillus cereus* may contain only one *cot* protein. However, using antisera to *cotA, cotB, cotC, cotD, cotE* and *cotF* in our collection it would be possible to identify homologous or cross-reacting coat proteins from the coats of spore formers and then clone the genes by reverse genetics.

Spores according to the invention could also be used with adjuvants. These might include cholera toxin, chitosan or aprotonin.